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Commentary

TaqMan Real-time PCR versus Four Conventional PCR Assays for Detection of Apple Proliferation Phytoplasma

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Abstract. A recently developed TaqMan real-time PCR assay for detection of apple proliferation phytoplasma was evaluated in comparison to four conventional PCR-based methods with the aim to assess its potential for research and routine applications. All five protocols were tested in parallel on the same DNA isolates obtained from orchard trees. The performance of the methods was evaluated by means of sensitivity, specificity, susceptibility to inhibition, handling effort, testing time, assay expenses, and potential risk for operator and environment. Compared to the conventional PCR methods, the TaqMan real-time PCR procedure combined the highest test sensitivity with the highest test specificity and was, above all, not susceptible to PCR inhibition. Furthermore, TaqMan real-time PCR had the simplest and fastest testing process, involving a minimum of handling steps. Its disadvantage is the high cost of consumables and reagents, exceeding that of a standard PCR procedure up to four-fold. However, the higher material costs could be compensated by considerably lower personnel costs and by saving expenses for hazardous waste disposal. Due to the simple testing procedure and the output of results as numeric data the TaqMan real-time PCR assay has a high potential for automation, and seems to represent the currently most suitable method for large-scale testing procedures.

Key words: diagnosis, *Malus domestica*, PCR inhibition, TaqMan real-time PCR, test sensitivity, test specificity

Abbreviations: AP, apple proliferation; NK, negative control; NTC, no-template control; PK, positive control.

Introduction

Apple proliferation (AP) is a disease of apple trees that poses a potential epidemic threat in some apple growing areas of central and southern Europe (Frisinghelli et al., 2000). This incurable disease can cause considerable economic losses by inducing a decrease of size, quality and overall yield of fruit (Seemüller et al., 1998a; Frisinghelli et al., 2000). Its causal agent is the AP phytoplasma, which is classified as a quarantine organism in Europe (EPPO, 2004) and North America (NAPPO, 2004). The pathogen is restricted to the phloem tissue of the plant and transmission is possible through infected propagation material and sap-sucking

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insect vectors of the genus *Cacopsylla* (Frisinghelli et al., 2000; Tedeschi et al., 2004).

Phytoplasmas (class Mollicutes) are small plant-pathogenic bacteria, which lack a cell wall and are known to cause a variety of plant diseases (Seemüller et al., 2002). Phytoplasma diagnostics prove difficult due to the impossibility of culturing these bacteria in cell-free media and because of their low titer and uneven distribution in woody plants (Seemüller et al., 1984; Ahrens and Seemüller, 1992; Waterworth et al., 1999; Errea et al., 2002). Assays based on polymerase chain reaction (PCR) are thus considered as the most effective tools to detect phytoplasmas in plant material (Deng et al., 1991; Ahrens and Seemüller, 1992; Lee et al., 1993; Lorenz et al., 1995; Smart et al., 1996; Green et al., 1999; Brzin et al., 2003). Thus far, a number of PCR based methods for diagnosis of AP phytoplasma are known. The majority of them employ oligonucleotide primers selected from the 16S ribosomal RNA gene and the 16S-23S intergenic spacer region. Some of the primers can be used for universal amplification of phytoplasmas, while others are specific for the 'AP phytoplasma group' (Ahrens and Seemüller, 1992; Jarausch et al., 1994; Lee et al., 1995; Lorenz et al., 1995; Smart et al., 1996; Seemüller et al., 1998b; Heinrich et al., 2001). Only a few diagnostic procedures allow exclusive and specific detection of AP phytoplasma, (*i*) by using primers based on a conserved chromosomal fragment of the AP phytoplasma (Jarausch et al., 2000), (*ii*) by employing a specific capture probe in a PCR ELISA assay (Poggi Pollini et al., 1997; 1999) or (*iii*) by performing a real-time PCR test with a specific TaqMan probe (Baric and Dalla Via, 2004).

Nowadays, real-time PCR has achieved wide acceptance in research and diagnostic laboratories (reviewed in Mackay et al., 2002 and Mackay, 2004). The reasons for the expanding popularity of real-time PCR are not only its elevated detection sensitivity but also its short analysis time and high automation capability. In contrast to conventional PCR, which requires laborious post-PCR handling steps for amplicon evaluation, such as gel electrophoresis or ELISA-like systems, real-time PCR combines amplification and detection in a closed system, thus minimizing the risk of carry-over contamination.

The aim of the present study was to evaluate the TaqMan real-time PCR assay for detection of AP phytoplasma (Baric and Dalla Via, 2004) in comparison to four conventional PCR-based methods in order to find the approach with the highest potential for large-scale testing procedures. The five different diagnostic protocols were tested in parallel on the same nucleic acid isolates, and the performance of the methods was evaluated by means of sensitivity, specificity, susceptibility to inhibition, handling, testing time, test expenses and potential risk for operator and environment.

Materials and Methods

A TaqMan real-time PCR assay and four conventional PCR assays for the detection of AP phytoplasma were evaluated by testing 162 DNA isolates in parallel. Single DNA extracts were prepared from each of 74 different apple trees, which were sampled in various orchards in the Province of Bozen/Bolzano (northern Italy) and an orchard in the Province of Venice. One DNA extract was obtained from an

Table 1. Primers and probes (names in italics) employed in the present study.

Detection assay		Sequences of primers and probes	Specificity	Reference
TaqMan real-time PCR	qAP-16S-F:	5′-CGAACGGGTGAGTAACACGTAA-3′	AP phytoplasma	Baric and Dalla Via, 2004
	qAP-16S-R:	5′-CCAGTCTTAGCAGTCGTTTCCA-3′		
	qAP-16S[a]:	5′-**FAM**–TAACCTGCCTCTTAGACG-3′		
	qMd-cpLeu-F:	5′-CCTTCATCCTTTCTGAAGTTTCG-3′	*M. domestica*	
	qMd-cpLeu-R:	5′-AACAAATGGAGTTGGCTGCAT-3′		
	qMd-cpLeu[a]:	5′-**VIC**–TGGAAGGATTCCTTTACTAAC-3′		
PCR fU5/rU4	fU5:	5′-CGGCAATGGAGGAAACT-3′	Phytoplasmas in general	Ahrens and Seemüller, 1992; Lorenz et al., 1995
	rU4:	5′-GAAGTCGAGTTGCAGACTTC-3′		
PCR fO1/rO1	fO1:	5′-CGGAAACTTTTAGTTTCAGT-3′	Fruit tree phytoplasmas	Lorenz et al., 1995
	rO1:	5′-AAGTGCCCAACTAAATGAT-3′		
PCR AP5/AP4	AP5:	5′-TCTTTTAATCTTCAACCATGGC-3′	AP phytoplasma	Jarausch et al., 2000
	AP4:	5′-CCAATGTGTGAAATCTGTAG-3′		
PCR ELISA	fO1:	5′-CGGAAACTTTTAGTTTCAGT-3′	AP phytoplasma	Lorenz et al., 1995; Poggi Pollini et al., 1999
	rO1:	5′-AAGTGCCCAACTAAATGAT-3′		
	APbiot1:	5′-**Biotin**–CGATGAAATATTTAGGTATG-3′		

[a]TaqMan MGB Probe with a non-fluorescent quencher dye at the 3′ end (Applied Biosystems, USA).

experimentally AP-infected plant maintained in the greenhouse. In addition, ten trees grown in an orchard close to Bozen/Bolzano were sampled at different parts of roots and branches, and up to nine DNA extracts were prepared for each plant.

Sampling was carried out during the growing season. Total nucleic acid was isolated from freshly prepared phloem tissue according to the simplified CTAB protocol described by Maixner et al. (1995) or by using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). From each apple tree included in the present study, phloem tissue was prepared out from three different parts of roots and approximately 300 mg was pooled for DNA isolation. From ten plants additional DNA extracts were obtained from single parts of roots and branches. Quantification of DNA was done by spectrophotometry at 260/280 nm or by using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Oregon, USA) in combination with a TECAN GENios Plate Reader (Tecan, Männedorf, Switzerland).

In addition to the (*i*) real-time PCR TaqMan assay for AP phytoplasma detection (Baric and Dalla Via, 2004), the following conventional PCR assays (combined either with gel electrophoresis of the amplified product or an ELISA-like system) were used in the present study: (*ii*) PCR with the universal phytoplasma primer pair fU5/rU4 (Ahrens and Seemüller, 1992; Lorenz et al., 1995), (*iii*) PCR with the specific primer set for fruit tree phytoplasmas fO1/rO1 (Lorenz et al., 1995), (*iv*) PCR with the specific primer combination for AP phytoplasma AP5/AP4 (Jarausch et al., 1994; 2000) and (*v*) PCR ELISA employing an AP-specific capture probe (Poggi Pollini et al., 1997; 1999) (Table 1).

Multiplex TaqMan real-time PCR reactions for a simultaneous detection of AP phytoplasma and its host plant were carried out in a total volume of 20 µl containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nM of both qAP-16S primers, 100 nM of both qMd-cpLeu primers, 200 nM of each probe and 2 µl of template DNA (Baric and Dalla Via, 2004). Each sample was amplified in duplicate in MicroAmp optical 96-well plates using the automated ABI PRISM 7000 Sequence Detection System (Applied Biosystems). PCR was initiated with two incubation steps: 2 min at 50°C (activation of AmpErase UNG; Applied Biosystems), 10 min at 95°C (activation of AmpliTaq Gold DNA polymerase; Applied Biosystems), followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Threshold cycles for each PCR reaction were calculated with ABI Prism 7000 SDS Software (version 1.0; Applied Biosystems).

The three conventional PCR assays fU5/rU4, fO1/rO1 and AP5/AP4 were performed in 20 µl reaction volumes that contained 1 µM of each primer, 200 µM dNTPs, 2.5 mM $MgCl_2$, 1 U HotMaster *Taq* Polymerase (Eppendorf, Hamburg, Germany) and 2 µl of DNA template. Amplification reactions were run on the Mastercycler Gradient (Eppendorf) or the GeneAmp PCR System 2700 (Applied Biosystems) under the following conditions: 2 min initial denaturation at 94°C, 40 cycles with 30 s at 94°C, 30 s at 55°C (fU5/rU4), 54°C (fO1/rO1) or 50°C (AP5/AP4) and 60 s at 65°C followed by 2 min of final elongation at 65°C. PCR amplification products were separated on ethidium bromide stained 1.5% agarose gels and visualized on the Gel Documentation System GelDoc 2000 (Bio-Rad Laboratories, Hercules, CA, USA).

The first step of the PCR ELISA protocol involved incorporation of digoxigenin-11-dUTP using the PCR ELISA DIG-Labelling Kit (Roche Applied Science,

Mannheim, Germany). 20 µl reaction volumes contained 0.5 µM of each of the primers fO1/rO1, 200 µM dATP, dCTP and dGTP, 190 µM dTTP and 10 µM DIG-dUTP, 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA Polymerase and 2 µl of DNA template. Amplification reactions were run on the GeneAmp PCR System 2700 under the following conditions: 5 min initial denaturation at 95°C, 38 cycles with 30 s at 95°C, 45 s at 54°C and 1 min 15 s at 72°C followed by 2 min of final elongation at 72°C. A hybridization-based assay implemented in the PCR ELISA DIG Detection Kit (Roche Applied Science) was used for subsequent detection of DIG-labeled products. First, 5 µl of each digoxigenin-labled PCR product was denaturated and hybridized with the biotin-labeled capture probe APbiot1 (Poggi Pollini et al., 1999) (Table 1). The concentration of the probe was 20 pmol per ml of denaturation buffer. After 3 hours of incubation at 37°C in a streptavidin-coated 96-well plate and shaking at moderate speed, each well was thoroughly rinsed (three times) with washing solution. Then the digoxigenin-specific anti-serum was added and incubated for 30 min. After a further washing step ABTS substrate solution was added and also incubated for 30 min. Photometric measurements were done on the TECAN GENios Plate Reader (Tecan) by reading the absorbance at a wavelength of 405 nm and a reference wavelength of 492 nm. Cut-off values were computed by multiplying the extinction of the negative control of each respective run by 2.5. A result was considered as PCR ELISA positive when the extinction was equal or higher than the according cut-off value.

Two DNA isolates were additionally tested by a nested PCR ELISA. The DIG-labeling reaction was preceded by an amplification reaction with universal phytoplasma primers P1/P7 (Deng and Hiruki, 1991) and the 50-fold diluted PCR product of this reaction was further used as template for PCR ELISA by applying the same procedure as described above.

Twenty-eight nucleic acid isolates that tested positive for AP phytoplasma with all five methods were selected to prepare a dilution series (100-fold, 1,000-fold and 10,000-fold dilution). The initial total DNA concentration of these extracts ranged from 3 to 100 ng/µl. Diluted DNA samples were tested using all five detection assays in parallel and applying the same conditions as described above.

Sensitivity (= true positives / [true positives + false negatives]) was computed for each of the five diagnostic assays by using data obtained with original DNA extracts as well as the dilution series, while specificity (= true negatives / [true negatives + false positives]) was calculated by using only data from original DNA extracts. In order to assess the percentage of PCR inhibition for each assay, the number of samples testing positive only after a 20-fold dilution of the original DNA extract was divided by the overall number of positive results found with that assay.

Workflow sequences were outlined for each diagnostic method in order to directly compare handling and testing duration. Finally, the expenses of each diagnostic assay were calculated based on list prices for reagents and consumables valid in the USA and list prices valid in Italy. For cost analysis it was presumed that (*i*) all reactions are performed in 20 µl volumes, (*ii*) 45 samples are tested in duplicate in 96-well plates, and (*iii*) two positive controls, two negative controls and two no-template controls are included. Personnel costs can affect the overall

Table 2. Comparison of the performance of five PCR-based detection assays for AP-phytoplasma diagnosis.

	N positive results	N negative results	N false positive or inconsistent results	Sensitivity	Specificity	Inhibition
TaqMan real-timePCR	119	43	-	100% (119/119)	100% (43/43)	0% (0/119)
PCR fU5/rU4	115	45	2[a]	96.6% (115/119)	95.6% (43/45)	11.3% (13/115)
PCR fO1/rO1	115	47	-	96.6% (115/119)	100% (43/43)	32.2% (37/115)
PCR AP5/AP4	114	48	-	95.8% (114/119)	100% (43/43)	13.2% (15/114)
PCR ELISA	117	42	2 + 1[b]	99.2% (117/118)	95.5% (42/44)	10.3% (12/117)

[a]False positive results caused by environmental bacteria (see Baric and Dalla Via, 2005).

[b]Inconsistent results (Sample 1: overall test result: negative, PCR ELISA: 2 × positive, 1 × negative; Sample 2: overall test result: negative, PCR ELISA: 2 × positive, 2 × negative; Sample 3: overall test result: positive, PCR ELISA: 1 × positive, 1 × borderline, 3 × negative.

assay-cost in a variable manner depending on testing duration and man-hours involved, as well as salary variability between different countries. Consequently, the higher the hourly wages, the more expensive an assay with long testing duration. Therefore, for reasons of comparison, personnel costs per assay were calculated by multiplying the testing duration by the hourly rates of a technical assistant, which was set at € 50.00 (US $ 59.55 at exchange rate as of submission date).

Results

Sensitivity

Of the 162 DNA isolates tested in parallel, 119 were found positive with real-time PCR, 117 with PCR ELISA, 115 with fU5/rU4 and fO1/rO1, and 114 with AP5/AP4 (Table 2). All 119 DNA isolates testing positive with real-time PCR were considered as real positive results because 117 of them overlapped with PCR ELISA and the two remaining DNA isolates were confirmed as positive with a nested PCR ELISA. Accordingly, 43 samples were regarded as real negatives. The highest test sensitivity (100%) was thus found for the real-time PCR assay, while the lowest test sensitivity (95.8%) was found for the conventional PCR with primer combination AP5/AP4 (Table 2).

C_T-values of positive real-time PCR samples ranged from 14.98 to 33.44 (mean 21.46 ± 3.94 SD). None of the real-time PCR negative samples displayed fluorescence signal above the threshold line. Conventional PCR followed by gel electrophoresis sometimes resulted in very weak bands, which were considered as positive results. The extinction values of the positive results obtained with PCR ELISA ranged from 0.236 to 2.678 (and overflow) with a mean of 1.706 (± 0.680 SD), while the extinctions for negative samples ranged between 0.075 and 0.200 (mean 0.127 ± 0.033 SD). The extinctions for negative controls varied between 0.075 and 0.125.

The dilution series test of 28 initially positively tested DNA isolates resulted in typical real-time PCR amplification plots for all samples covering the entire dilution range (Table 3). The C_T-values ranged from 22.0 to 33.0 (mean 27.5 ± 2.83 SD) for 100-fold dilutions, from 25.3 to 36.1 (mean 31.0 ± 2.72 SD) for 1,000-fold dilutions and 29.4 to 40.0 (mean 34.8 ± 2.92 SD) for 10,000-fold dilutions of the original DNA isolates. Considerably lower test sensitivities were obtained with conventional PCR when using primer pair AP5/AP4: only 75% of the 100-fold dilutions and 3.6% (one sample) of the 10,000-fold dilutions tested positive (see Table 3 for summary of all results). Extinction values for positive PCR ELISA results ranged from 0.290 to 2.582 (mean 1.524 ± 0.625 SD) for 100-fold dilutions, from 0.211 to 2.600 (mean 1.321 ± 0.793 SD) for 1,000-fold dilutions and from 0.297 to 2.135 (mean 0.845 ± 0.544 SD) for 10,000-fold dilutions, while the extinction values for negative results varied from 0.079 to 0.212 (mean 0.150 ± 0.048 SD) for 1,000-fold dilutions and from 0.094 to 0.234 (mean 0.152 ± 0.045 SD) for 10,000-fold dilutions.

Table 3. Comparison of sensitivity of the five PCR-based detection assays for AP-phytoplasma using a dilution series of 28 positively tested DNA isolates obtained from field samples. The total DNA quantity of the original extracts ranged from 3 to 100 ng/µl.

	Dilution		
	100-fold	1,000-fold	10,000-fold
TaqMan real-time PCR	100% (28/28)	100% (28/28)	100% (28/28)
PCR fU5/rU4	100% (28/28)	92.9% (26/28)	53.6% (15/28)
PCR fO1/rO1	100% (28/28)	57.1% (16/28)	32.1% (9/28)
PCR AP5/AP4	75.0% (21/28)	35.7% (10/28)	3.6% (1/28)
PCR ELISA	100% (28/28)	89.3% (25/28)	53.6% (15/28)

Specificity

In the present study false positive or inconsistent results were obtained with two assays: PCR fU5/rU4 and PCR ELISA (Table 2). For two samples primer combination fU5/rU4 delivered weak bands of the same size as the specific product. As these amplicons were reproducible with the same primer set, but were not confirmed with any other assay, they were considered as false positive results. Moreover, one of these PCR products was sequenced and identified as a segment of the 16S rRNA gene of an environmental bacterium (Baric and Dalla Via, 2005). PCR ELISA gave inconsistent results with three DNA isolates. Two of these were considered as false positive results as the overall test result was negative and the results obtained with PCR ELISA were twice positive and once negative for one sample, and twice positive and twice negative for the second sample (Table 2). The specificity of PCR fU5/rU4 and PCR ELISA was found to be 95.6% and 95.5%, and thus lower in comparison to the other assays, which showed a specificity of 100%.

Inhibition

All test assays were initially performed with original DNA isolates, and when negative results were obtained, the test was repeated with a 20-fold dilution of the original DNA extract. The real-time PCR primer/probe set for AP phytoplasma detection was not affected by inhibitors as all the AP positive samples were found by using original DNA isolates. However, the segment of the *M. domestica* chloroplast DNA being amplified simultaneously and serving as an internal positive control showed atypical amplification plots in some of the AP negative samples (Figure 1, curve B) and in others the fluorescence signal did not even exceed the threshold line (Figure 1, curve C). When repeating the test with diluted DNA isolates these samples remained negative for AP phytoplasma but displayed typical amplification curves for the internal positive control (Figure 1, curve A). Therefore the possibility of PCR inhibition can be excluded and the diagnosis of uninfected plant material can be confirmed.

The conventional PCR assays were affected to a greater extent by PCR inhibition (Table 2). Although working with the same primer set, 10.3% of the AP positive samples were initially affected by inhibition in PCR ELISA, while 32.2% of the AP positive samples were affected in PCR with primers fO1/rO1. These

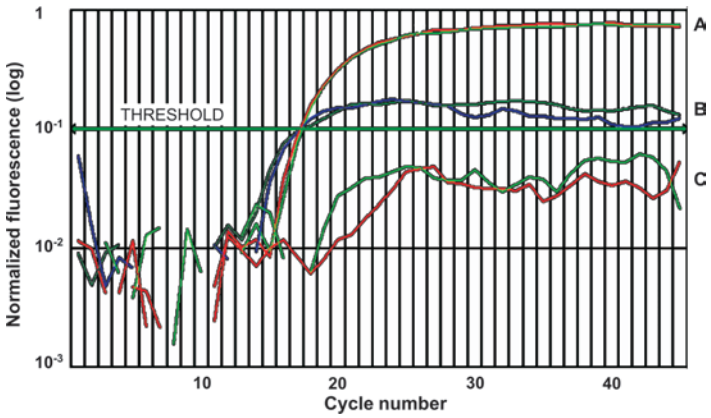

Figure 1. The effect of inhibition on the amplification efficiency of the internal positive control implemented in the TaqMan real-time PCR assay. Amplification plot unaffected by inhibition (A), moderate inhibition (B), and complete inhibition (C).

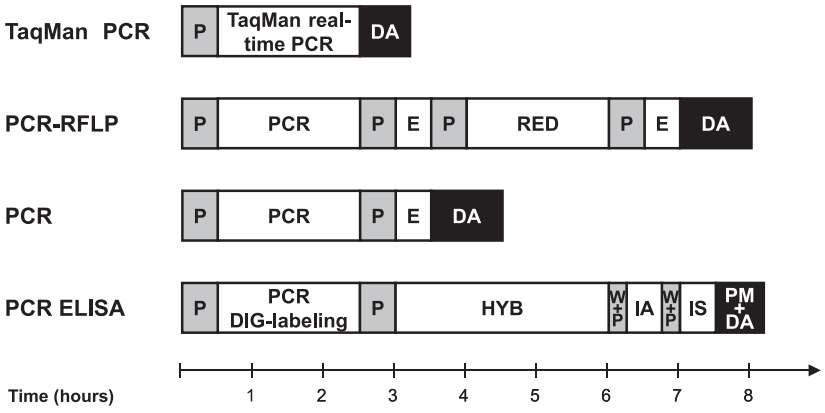

Figure 2. Workflow sequences of different PCR-based assays to detect AP-phytoplasma. Grey boxes denote work steps involving hands-on time by laboratory personnel, white boxes indicate steps processed solely by laboratory instrumentation and black boxes represent data analysis. DA, data analysis; E, electrophoresis; HYB, hybridization of probe to PCR product and immobilization on streptavidin-coated 96-well plate; IA, incubation with antiserum; IS, incubation with substrate solution; P, sample and reagent preparation for the next work step; PM, photometric measurement; RED, restriction enzyme digestion; W, washing step.

samples turned out positive when re-testing 20-fold dilutions of the original DNA extracts.

Test expenses, handling and testing time

The expenses for reagents and consumables employed for the different diagnostic assays ranged from US $ 1.34 to US $ 7.30 (€ 1.48 to € 8.30) per sample when testing 45 samples in duplicate in 96-well plates, and including two positive controls, two negative controls and two no-template controls (see Table 4). The shortest testing duration of 3:15 hours was found for the real-time PCR while 8:15 hours were needed to perform a PCR ELISA assay (Table 5). Real-time PCR showed the simplest and shortest workflow sequence requiring only a single handling

Table 4. Comparison of expenses for reagents and consumables for different PCR-based assays to detect AP phytoplasma. Calculations are based on list prices valid in the USA and list prices valid in Italy (in parentheses). Some companies offer reduced prices under special agreements, which were not considered herein.

	TaqMan real-time PCR		PCR-RFLP		PCR		PCR ELISA	
	US $	€	US $	€	US $	€	US $	€
PCR Reagents including primers (20 µl)[1]	183.78	*(242.11)*	23.81	*(27.36)*	23.81	*(27.36)*	140.93	*(147.65)*
PCR plate with cover[2]	7.15	*(8.75)*	5.03	*(5.09)*	5.03	*(5.09)*	8.97	*(9.05)*
Pipet tips[3]	11.90	*(12.70)*	35.70	*(38.10)*	23.80	*(25.40)*	35.70	*(38.10)*
Agarose gel and size standard[4]	-	-	7.52	*(8.65)*	7.52	*(8.65)*		-
Restriction enzyme with reaction tubes[5]	-	-	7.13	*(10.88)*	-	-		-
Low Range Agarose and size standard[6]	-	-	5.43	*(6.29)*	-	-		-
DIG Detection Kit with probe[7]	-	-	-	-	-	-	143.04	*(178.56)*
Expenses per 96-well plate with 45 duplicate samples, 2 PK, 2 NK and 2 NTC	202.83	*(263.56)*	84.62	*(96.37)*	60.16	*(66.50)*	328.64	*(373.36)*
Expenses per sample	4.51	*(5.86)*	1.88	*(2.14)*	1.34	*(1.48)*	7.30	*(8.30)*
Relative test expenses[8]	3.4	*(4.0)*	1.4	*(1.4)*	1.0	*(1.0)*	5.5	*(5.6)*
Personnel costs per assay[9]		*162.50*		*400.00*		*225.00*		*412.50*
Costs per assay including reagents/consumables and personnel		*426.06*		*496.37*		*291.50*		*785.86*
Expenses per sample including reagents/consumables and personnel		*9.47*		*11.03*		*6.48*		*17.46*
Relative test expenses[8] including reagents/consumables and personnel		*1.5*		*1.7*		*1.0*		*2.7*

[1]TaqMan real-time PCR: TAQMAN Mastermix (Applied Biosystems, $ 395.00 [€ 525.00] / 250 samples) and TaqMan MGB Probe (Applied Biosystems, $ 250.00 [€ 330.00] / 1625 samples). PCR-RFLP and PCR: HotMaster Mix (Eppendorf, $ 56.00 [€ 66.20] / 250 samples). PCR-ELISA: DIG Labeling Kit (Roche, $ 361.00 [€ 379.50] / 250 samples). Primers for all assays: MGB (average price of $ 12.00 [€ 10.00] for 1000 samples).

[2] Real-time PCR: 96-well optical plates with optical covers (Applied Biosystems, $ 715.00 [€ 875.00] / 100 plates with covers). PCR-RFLP, PCR and PCR-ELISA: 96-well plate and self-adhesive foil (Eppendorf, $ 87.00 [€ 99.10] / 25 plates and $ 155.00 [€ 113.00] / 100 foils), for PCR ELISA one additional plate without cover is needed for the post-PCR denaturation reaction.

[3]Filter Tips (Eppendorf, $ 11.90 [€ 12.70] / 96 tips).

[4]100 ml 1.5 % Molecular Biology Agarose (BioRad, $ 127.00 [€ 87.00] / 125 g); 8 slots Molecular Ruler (BioRad, $ 75.00 [€ 95.00] / 100 slots).

[5]Price is based on the assumption that half of the samples are positive (22 samples and positive control); Restriction Enzyme *Ssp*I, (New England Biolabs, $ 58.00 [€ 110.00]/ 500 U); reaction tubes 1.5 ml (Eppendorf, $ 39.00 / 500 tubes; [€ 33.00 / 1000 tubes]).

[6]50 ml 2 % Low Range Agarose (Bio-Rad Laboratories, $ 304.00 [€ 311.00] / 125 g), 4 slots Molecular Ruler (Bio-Rad Laboratories, $ 75.00 [€ 95.00] / 100 slots).

[7]DIG Detection Kit (Roche, $ 276.00 [€ 348.00] / 192 samples); biotinylated probe (MWG, $50.00 [€ 50.00] / 1000 samples).

[8]Test expenses relative to the least expensive test assay (PCR).

[9]Personnel costs per assay were calculated by multiplying the testing duration by the hourly rates of a technical assistant, which were assumed to be € 50.00.

Table 5. Summary of test characteristics of five PCR-based assays to detect AP phytoplasma in field samples.

	TaqMan real-time PCR	fU5/rU4 (with RFLP)	fO1/rO1 (with RFLP)	AP5/AP4	PCR ELISA
Sensitivity	100%	96.6%	96.6%	95.8%	99.2%
Specificity	100%	95.6%	100%[1]	100%	95.5%
Inhibition	none; IPC[2]	11.3%	32.2%	13.2%	10.3%
Handling	simple	laborious	laborious	medium	laborious
Testing duration	3:15 h	8:00 h	8:00 h	4:30 h	8:15 h
Potential hazard for operator and environment	none	ethidium bromide, UV, acrylamide, silver nitrate	ethidium bromide, UV, acrylamide, silver nitrate	ethidium bromide, UV	none
Potential for automation	high	low to medium	low to medium	low to medium	medium
Relative test expenses (RC)[3]	3.4 (4.0)	1.4 (1.4)	1.4 (1.4)	1.0 (1.0)	5.5 (5.6)
Relative test expenses (RCP)[4]	*1.5*	*1.7*	*1.7*	*1*	*2.7*

[1]Although in the present study no false positives were detected, such results are known from a previous study (Baric and Dalla Via, 2005). If the diagnostic procedure involves only primers fO1/rO1 we strongly recommend confirming the identity of the PCR products by RFLP

[2]IPC, internal positive control

[3]Test expenses relative to the least expensive test assay (PCR AP5/AP4) in the USA and in Italy (in parentheses) by including costs for reagents and consumables only

[4]Test expenses relative to the least expensive test assay (PCR AP5/AP4) in Italy by including costs for reagents, consumables and personnel

step. In contrast, PCR-RFLP or PCR ELISA demanded four handling steps involving laboratory personnel (see Figure 2).

After factoring in personnel costs, PCR with primers AP5/AP4 was the least costly test assay. The higher material costs for the real-time PCR were compensated by the lower personnel costs, so that its overall test expenses were only 1.5-fold higher than that of the least expensive assay. PCR ELISA emerged to be the most expensive test, with 2.7-fold higher overall costs in comparison to the most economic assay (Tables 4 and 5).

Discussion

An important step in the control of apple proliferation is fast and specific pathogen detection. Several PCR-based methods have thus been developed. However, validation of these methods usually involved a limited number of DNA isolates, which were either obtained from defined phytoplasma strains maintained in periwinkle, or from field samples with pronounced disease symptoms (Ahrens and Seemüller, 1992; Jarausch et al., 1994; 2000; Lorenz et al., 1995; Gundersen et al., 1996; Poggi Pollini et al., 1997; 1999; Heinrich et al., 2001). The present study is the first to evaluate five different PCR-based assays for AP phytoplasma detection in parallel by using DNA extracts from orchard trees in order to assess their potential for large-scale diagnosis and monitoring procedures.

One major problem in AP detection is the uneven distribution and typically low concentration of the pathogen in the aerial parts of the tree (Seemüller et al., 1984; Waterworth et al., 1999; Errea et al., 2002). As a result, even plants showing typical symptoms of AP disease may not test positive in a PCR detection test (Tedeschi and Alma, 2004). This problem may be overcome to some extent by careful sampling procedures, which should favor inclusion of root samples in order to detect latent AP infections (Carraro et al., 2004). In the present study real-time PCR was shown to have the highest test sensitivity, which became particularly obvious when analyzing serial dilutions of AP positive samples (Table 3). While real-time PCR successfully detected the pathogen in all samples over the entire dilution range, the performances of the conventional PCR assays varied considerably and displayed maximum test sensitivities ranging from 3.6% to 53.6% at 10,000-fold dilutions. Although test sensitivity did not differ as drastically when surveying undiluted or 20-fold diluted DNA isolates, 1.7% of the AP-positive samples were detected only with real-time PCR (and confirmed by a nested PCR-ELISA). One of the two DNA extracts was taken from the branch of a tree showing typical AP symptoms, and infection of this plant with AP phytoplasma was verified with all five diagnostic methods by testing six independent root-derived DNA samples.

In the present study real-time PCR demonstrated not only elevated sensitivity but also high test specificity. In contrast, PCR fU5/rU4 and PCR ELISA gave false positive results, each with two DNA isolates. It was not possible to identify the source DNA being responsible for the false positive results obtained with PCR ELISA. However, the false positive results with primers fU5/rU4 were caused by amplification of bacterial DNA present in nucleic acid isolates from root samples (Baric and Dalla Via, 2005). Unintentional amplification reactions can occur due to high levels of sequence homology of these primers to regions of the 16S rRNA

gene of environmental bacteria. Even though primer set fO1/rO1 delivered highly specific results with all samples surveyed, a previous study also indicated the possibility of amplifying unspecific products with this primer set when testing DNA isolated from roots (Baric and Dalla Via, 2005). Therefore, we highly recommend verification by RFLP analysis of positive test results when using primers fU5/rU4 or fO1/rO1.

It is widely recognized that DNA isolated from plant material may contain inhibitory compounds that can compromise the sensitivity and reliability of PCR assays (Green et al., 1999; Musetti et al., 2000; Langrell et al., 2001). Inhibitory effects on phytoplasma diagnosis have been assessed using DNA isolated from ESFY-infected *Prunus*, which should represent the most difficult sample situation (Heinrich et al., 2001). Our study showed that diagnostic assays for AP detection can also be compromised by the presence of inhibitory substances, though to different degrees. While AP phytoplasma detection by real-time PCR was not affected by PCR inhibition, primer set fO1/rO1 yielded 32.2% false negative results. This indicates that samples testing negative should be diluted and re-tested, which could be critical when testing samples with low pathogen titers. However, such measures would considerably increase expenses, handling time and duration of the testing procedure. It thus appears that the incorporation of an internal positive control, as implemented in the real-time PCR assay, is essential, as it allows one to immediately distinguish a negative test result caused by the absence of the pathogen from that caused by PCR inhibition.

Although test sensitivity and specificity are important considerations when evaluating the reliability of a diagnostic assay, additional criteria, such as applicability, cost, labor requirements and potential risk for operator and environment, should not be disregarded. A direct comparison of workflow sequences revealed real-time PCR as the simplest and shortest procedure that involves only a single sample preparation step (Figure 2). For the other assays, post-amplification steps, such as gel electrophoresis, hybridization with specific probes and immunoenzymatic determination of amplicons or digestion with restriction endonucleases are required. These procedures not only involve more handling steps and longer testing time, but also bear an increased risk of crossover contamination, which can be completely avoided in real-time PCR. In addition, detection of DNA fragments by gel electrophoresis involves hazardous substances (acrylamide gel) or mutagenic (ethidium bromide) and toxic (silver nitrate) stains.

The calculation of material costs showed that PCR AP5/AP4 combined with agarose gel electrophoresis was the most economic assay. However, due to the potentially high numbers of samples falsely testing negative (due to some extent to inhibitory effects) and the need for a second round of testing with diluted DNAs, costs for reagents and consumables would rise. Moreover, the demand for re-testing would increase the testing duration and double personnel involvement. Although material costs for real-time PCR were 3.4-fold (USA) to four-fold (Italy) higher than that of the most economic assay, these were compensated by the lower personnel costs and the high test accuracy of the real-time PCR assay. In addition, due to the omission of toxic substances, expenses incurring for hazardous waste disposal can be avoided. Although these costs do not apply to the

PCR ELISA procedure, it remains the most expensive assay, considering both reagents and consumables, and labor.

The present study shows that real-time PCR for the detection of AP phytoplasma is the most accurate and most rapid testing procedure, compared to the other assays surveyed. The possibility for automation given by the small number of handling steps and the output of results as numerical data bears a high potential for application in large-scale testing procedures such as certification programs or pathogen surveys. The current disadvantage of this technology is surely the high purchase price of the specialized equipment required and the relatively high material costs. However, real-time PCR is being applied in more and more diagnostic procedures (Mackay et al., 2002; Mackay, 2004). Therefore it is likely that this technology will become more affordable in the near future, and thus become increasingly present as standard equipment in many phytopathological laboratories (Schaad et al., 2002; Hollomon, 2003).

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